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Chapter 4

Salivary Diagnostics

Karolina Elżbieta Kaczor-Urbanowicz

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Abstract

Salivary diagnostics plays an important role in the early detection and prevention of many oral and systemic diseases in a fast and noninvasive way. Saliva collection is an easy, repeatable and inexpensive diagnostic source that can be used for both diagnosis and real-time monitoring of various human diseases. In the near future, many developed and validated salivary biomarkers have the potential to reach the clinical practice. Five diagnostic "omics" constituents of saliva include proteomics, transcriptomics, metabolomics, microbiomics and microRNAs. Based on them, the newly emerging technologies of salivary diagnostics are developed that include RNA-sequencing, point-of-care technologies and liquid biopsy. They have potential to enable screening, early detection, prognosis and monitoring of various human diseases. The recent developments broadened the salivary diagnostic approach from the oral cavity to the whole physiological system, thus toward personalized individual medicine applications.

Keywords: saliva, diagnostics, oral cancer, RNA-sequencing, point-of-care, liquid biopsy

1. Introduction

Saliva is an encouraging medium to be used in the early detection, diagnosis and monitoring of oral and systemic diseases, specifically for the purpose of personalized medicine by incorporating point-of care technology platforms in the clinical settings. Though, saliva collection is easy, fast, cheap, safe, does not require specialized equipment and can be performed at home [1]. The normal daily production of saliva varies between 0.5 and 1.5 l. Saliva is an acidic biofluid, derived from the three major salivary glands (parotid, submandibular, sublingual) as well as from minor glands (labial, buccal, lingual, and palatal tissues). It is composed in vast majority of water (99%), while other constituents occur in trace amounts, including proteins and both inorganic (sodium, potassium, calcium, magnesium, chloride, etc.) and organic

constituents (amylases, peroxidase, lipase, mucins, lysozyme, lactoferrins, cystatins, hormones, etc.) [2]. Salivary composition is very diverse (RNA, DNA, proteins, metabolites, and microbiota), and may be utilized for diagnostic purposes. Most importantly, salivary components may vary in their concentrations and levels depending on the individual's health or disease status. Thus, real-time monitoring of salivary data can provide useful translational clinical applications in the detection of various human oral and systemic diseases. The development of the recent technologies based on salivary diagnostics will help to introduce screening programs to enable early detection and monitoring of the disease [3].

Saliva is an important biofluid with lots of various biological functions including lubrication, chewing, swallowing, sensation, digestion and protection of oral mucosa against biological, mechanical, and chemical factors as well as infections [2].

2. Salivaomics

Currently, there are known five major diagnostic toolboxes of saliva "Salivaomics": proteomics (the study of proteins), transcriptomics (the study of RNAs), metabolomics (the study of metabolites), microRNA (the study of microRNAs) and microbiome (the study of microbiota) [1, 2] (**Figure 1**).

2.1. Proteomics

The proteomics is the large-scale screening for proteins, their expression, modifications, and interactions by using high-throughput approaches [4, 5]. Proteins can indicate various physiological and pathological states of the current health condition or specific disease. The recent advancements in proteomics contributed to the development of new non-invasive technologies.

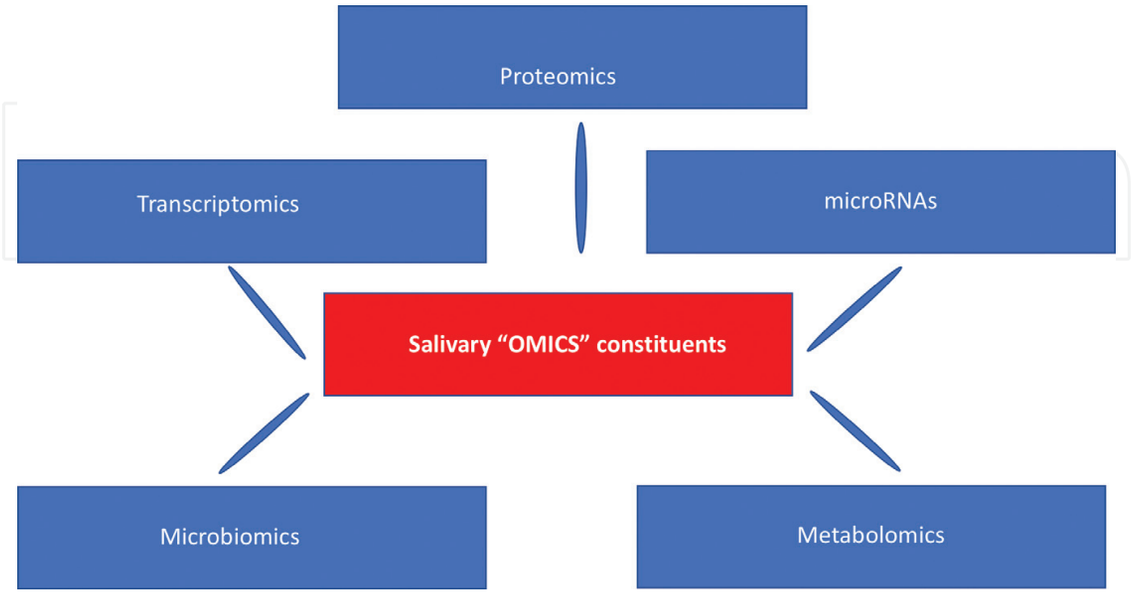

Figure 1. Diagnostic toolboxes of saliva.

The currently accepted gold standard methods for proteomic analyses include: triple depletion of high abundance proteins (removal of albumins, alpha amylase and immunoglobulins), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), two-dimensional gel electrophoresis (2-DE), mass spectrometry (label free qMS), ELISA (enzyme-linked immunosorbent assay) and Western blotting [2, 6]. Other advanced methods include electrospray ionization (ESI), matrix-assisted laser desorption ionization (MALDI), quadrupole/linear ion trap, time-of-flight (TOF), quadrupole TOF (QTOP), Fourier transform ion cyclotron resonance (FT-ICR), or the OrbiTrap, MS/MS, MALDI-MS or targeted HPLC-ESI-MS/MS [7].

Recently, a great focus has been put on identification of salivary protein biomarkers for various human oral and systemic diseases such as: pancreatic cancer, Sjogren's syndrome, oral cancer, lung cancer, orthodontically induced root resorption, etc. [2, 5]. The proteomics delivers an alternative ideal and non-invasive diagnostic tool, more sensitive, and safer for detecting the disease status. In addition, the depletion of high abundance proteins from saliva contributes to significant increase in the detectability of less abundant salivary proteins [5, 8]. There are known three major methods of high-abundance protein removal [9]: enzyme-substrate absorption method used for alpha-amylase affinity removal [8], immunodepletion method and combinatorial peptide ligand library (CPLL) [10].

Proteomic analysis of saliva is commonly used in the diagnostics of oral diseases as well as general health disorders such as oral candidiasis [11], oral squamous cell carcinoma (OSCC) [12], glossodynia [13], head and neck squamous cell cancer [14], Sjögren's syndrome [15], HIV [11], autism [16], fibromyalgia [17], breast cancer [18], lung cancer, melanoma [19] or pancreatic cancer [7].

Various mediators associated with oral cancer are released from cells due to malignant conditions and have been analyzed in saliva samples, like cytokines, chemokines, interferon-gamma (IFN-γ), interleukins (IL-1β, IL-6 and -8, Il-4 and -10), tumor necrosis factors (TNF-α), transforming growth factor-beta-1 (TGF-β1), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and endothelin [20]. In case of oral squamous cell carcinoma, elevated levels of NF- κB-dependent cytokines have been observed in saliva [21]. Other potential protein biomarkers include IL-6 and S100A9 [22] or BGH3, MMP9 and PDIA3 [23]. In addition, increased salivary expression levels of MMP1 and MMP3 in OSCC patients can indicate more advanced stage of disease [12], while adenosine deaminase might be indicative of early stage of oral tongue cancer [24].

2.2. Transcriptomics

Transcriptomics (gene expression profiling) is the quantitative study of an organism's transcriptome, all RNA transcripts present in a cell. The information is recorded in the genome and expressed through transcription. These data can be used for capturing marked changes in expression levels of specific genes in the detection of various human diseases [2, 25]. Transcriptomics encompasses a great diversity of RNA species including messenger RNAs (mRNAs), long non-coding RNAs (lncRNAs), and small RNAs such as microRNAs (miRNAs), transfer RNAs (tRNAs), piwi-interacting RNA (piRNA), etc. The mRNAs play an important

role in carrying information for making proteins while noncoding RNAs have different functions [25]. In turn, the microRNAs (miRNAs) are a group of small non-coding RNAs that regulate mRNA through sequence-specific binding to the UTR [26]. The miRNAs are involved in various biological processes.

The common practice in identification of salivary transcriptomic biomarkers is microarray technology. However, this technology is currently replaced by the newer one, RNA-sequencing (RNA-Seq).

2.2.1. RNA sequencing

RNA-sequencing (RNA-Seq) has been the newly developed method for characterizing the full human transcriptome. RNA-sequencing (RNA-Seq) has a wide variety of applications, but no single analysis pipeline can be used in all cases and for all biofluids [27]. Specifically, RNA-Seq of saliva is challenging, including difficult RNA isolation step to enhance yield of salivary exRNA step as well as laborious RNA-Seq small and large library construction stage, inclusion of spike in standards and controls, RNA-sequencing, data storage and data analysis. Therefore, currently available literature regarding salivary RNA-Seq is scarce [28–30].

RNA-sequencing is a rapidly progressing transcriptome profiling that uses deep-sequencing technologies and becoming the major tool in analyzing gene expression [31]. This is a new high-throughput method for both mapping and quantifying transcriptomes. It provides more detailed information about the levels of transcripts and their isoforms than other methods [32]. In general, a total RNA is converted to a library of cDNA fragments with adaptors attached to one or both ends. Afterwards, a sequencing of the molecule is performed, with or without amplification, that provides short sequences from one (single-end sequencing) or both ends (pair-end sequencing). The reads are typically 30–400 bp, depending on the DNA-sequencing technology used. Revealing the transcriptome is crucial for investigating the functional elements of the genome, the molecular constituents of cells and tissues, and also for understanding development and disease [32].

2.2.1.1. Advantages of RNA sequencing

RNA- Seq (RNA sequencing) has clear advantages over existing approaches and is believed to become the best method for analyzing transcriptome in the near future.

Firstly, RNA-Seq can be used for detection of both known (corresponding to existing genomic sequences) and novel transcripts, thus enabling identification of new organisms with unidentified yet genomic sequences. RNA-Seq is used for precise localization of transcription boundaries, to a single-base resolution. Furthermore, this method can be used for examining transcripts of great complexity as it provides useful information about the connectivity of exons as well as sequence variations in the transcribed regions [33–34].

Secondly, RNA-Seq has very low, if any, background signal. This feature differentiates it significantly from microarray platforms as it can be uniquely mapped to the genome regions of interests [35].

RNA-Seq has also significant benefits over array-based technologies for detecting expression quantitative trait loci (eQTLs). Though, it can identify different transcript variants and enable quantification of allele-specific expression within an individual to increase association mapping [27, 36, 37].

Finally, it has a highly accurate and large dynamic range of expression levels with high reproducibility rates while using less RNA sample and at a much lower cost than either tiling arrays or large-scale Sanger EST Sequencing [33, 35].

2.2.1.2. Disadvantages of RNA sequencing

The major problems of RNA-Seq technology are associated with RNA isolation and cDNA library construction as it includes several manipulation stages, which can complicate profiling of transcripts of various lengths [32]. This causes the variability in the measurements, influenced by the technical noise [27].

In addition, longer RNAs have to be fragmented into smaller pieces (200–500 bp) to be compatible with most deep-sequencing technologies using RNA or cDNA fragmentation methods (RNA hydrolysis or nebulization, DNase I treatment, etc.) [33, 35]. These sequencing procedures include a number of steps (RNA fragmentation, cDNA synthesis, adapter ligation, PCR amplification, bar-coding, and lane loading) that might introduce biases into the resulting data [27].

Another key consideration concerning library construction is whether or not to construct strand- specific libraries [33, 38], that provide information about the orientation of transcripts, essential for transcriptome annotation. However, strand-specific libraries are very time-consuming to produce [39, 40].

RNA-Seq has also bioinformatics challenges associated with storage, retrieval and processing large amounts of data. The alignment of long RNA-Seq reads is also complicated due to non-unique mapping to multiple locations in the genome [32].

Also, to detect a rare transcript or variant, more sequencing depth is needed. In general, the larger the genome, the more sequencing depth is required for adequate coverage, which brings greater cost [32].

Although RNA-Seq is still in the early stages of use, it has clear advantages over previously developed transcriptomic methods such as microarray profiling [32]. Specifically, salivary RNA biomarker transcripts of IL8, IL1B, DUSP1, HA3, OAZ1, S100P, and SAT yielded high sensitivity (91%) and specificity (91%) in distinguishing OSCC from the controls [41]. Also, salivary IL6 mRNA and IL-8 may serve as potential biomarkers for diagnosis of OSCC [42, 43].

2.2.2. Difficulties in salivary RNA-sequencing and bioinformatic analysis

RNA-sequencing (RNA Seq) of saliva is challenging compared to other biofluids such as blood or urine. RNA Sequencing of salivary samples is associated with several problems such as inadequate technique of RNA isolation, improper stabilization of RNA or RNA library construction [44–48]. Till now, there were also no established guidelines how to bioinformatically process the salivary RNA-Seq data. The recent paper by Kaczor-Urbanowicz et al. gives the

recommendations for bioinformatics analysis of salivary RNA-Seq data that differs from other biofluids (blood, urine, etc.) as saliva contains the majority of microbial content, while other physiological fluids are considered to be sterile [48]. Thus, it is recommended to use quite stringent and sensitive criteria, while working with salivary RNA-Seq data to avoid erroneously mapped bacterial reads to the human genome, and to prevent problems with their further annotation to human RNA databases. In addition, the specific sequence of alignment steps and the stringency parameters associated with processing of RNA Sequencing data can grossly increase the final data quality [48].

2.3. Micro-RNA-omics

MicroRNAs (miRNAs) are short, single-stranded RNAs that are about 21 nucleotides in length. Their function is to regulate gene expression. Like other types of RNA, miRNAs are transcribed from DNA, but they do not participate in protein translation. They are non- coding RNAs, in which each primary transcript (pri-miRNA) is processed into a pre-miRNA and finally into functional miRNA [49]. Mature miRNA are involved in various biological processes such as cell growth, differentiation, apoptosis, stress and immune response or glucose secretion [50–52]. Studies on miRNA dysregulation in various human diseases have risen rapidly in recent years, including those in cancer, heart disease as well as type II diabetes mellitus and its complications, such as endothelial and vascular smooth muscle cell dysfunction, cardiomyopathy and nephropathy [53–55]. Most importantly, salivary microRNAs (miRNAs) (miR-9, miR-134 and miR-191) can be used as potential biomarkers for head and neck squamous cell carcinoma [56]. The reduction in salivary expression profiles of miR-125a and miR-200a was observed in OSCC patients compared to healthy people [57]. In turn, miR-31 increases in OSCC patients, specifically in saliva, where it rises even more than in plasma [58].

2.4. Metabolomics

Metabolomics is the study of small molecular metabolites of living tissues, mostly metabolic intermediates such as carbohydrates, lipids, amino acids, nucleic acids, etc. [1]. The major metabolomic technologies include high-performance liquid chromatography-mass spectrometry (HPLC-MS), two-dimensional gas chromatography MS and nuclear magnetic resonance spectroscopy in conjunction with pattern recognition methods [2].

Salivary metabolites are involved in many biological processes as well as pathogenesis of various diseases such as periodontal diseases, renal diseases, hepatocellular carcinoma and colorectal cancers [59] as well as oral cancer [60]. In case of oral leukoplakia, an upregulation of putrescine, 8-hydroxyadenine and 5,6-dihydrouridine in OSCC can be indicative of increased risk for malignant transformation [61].

2.5. Microbiomics

Microbiomics include study of bacteria, archaea, protists, fungi and viruses. Microbial profiling (Human Oral Microbe Identification Microarray) of salivary microbiome in early resectable pancreatic cancer revealed that Neisseria elongata and Streptococcus mitis were successfully

developed with 96.4% sensitivity and 82.1% specificity [62]. Currently, newer microbiome-based technologies have been developed such as RNA or DNA sequencing [1]. In addition, two microbial biomarkers, Firmicutes (especially Streptococcus) and Actinobacteria (especially Rothia) were significantly decreased in oral cancer compared to healthy controls [63]. Finally, Furquim et al. reported that patients with Fanconi anemia (FA) are at higher risk of developing OSCC than the general population, especially after the hematopoietic stem cell transplantation [64].

3. Modern technologies in salivary diagnostics

3.1. Salivary liquid biopsy

Recently, a new trend appeared to reveal emerging role of "liquid biopsy" as identification method of biomarkers in various cancers. Liquid biopsy tests are non-invasive biofluid tests (serum, urine, saliva) that detect circulating tumor cells (CTCs) and fragments of tumor DNA shed into the bloodstream by cells undergoing apoptosis or necrosis [3].

The role of liquid biopsy markers including circulating tumor cells, circulating RNAs (miRNA, lncRNAs and mRNAs), cell-free proteins, peptides and exosomes has been currently investigated as non-invasive cancer biomarkers in different biofluids such as blood, urine, saliva and seminal plasma. Liquid biopsies hold great promise for personalized medicine due to the fact that they enable multiple non-invasive global sampling resulting in longitudinal assessment of the primary and metastatic tumors. Molecular profiling of circulating molecules (proteomic, transcriptomic, genomic, metabolomics, microRNAs) contributed to the successful application of several non-invasive multi-marker tests in the clinic [65].

Nowadays, liquid biopsy enables a variety of clinical and investigational applications such as early detection, assessment of molecular heterogeneity of general disease, monitoring of tumor dynamics (in melanoma, breast, ovarian or colon cancers), identification of genetic determinants for targeted therapy, evaluation of early treatment response, monitoring of minimal residual disease or assessment of resistance evolution in real time [66].

The most common technologies of liquid biopsy include detection and quantification of ctDNA (circulating tumor DNA) in blood such as Sanger sequencing, pyrosequencing, next generation sequencing, PCR-based technology, high-performance liquid chromatography (HPLC), mutant-enriched liquid chips, amplification refractory mutation system (ARMS), beads, emulsion, amplification and magnetics (BEAMing), pyrophosphorolysis-activated polymerization (PAP) [2, 66] or electric field-induced release and measurement (EFIRM) [67, 68]. The current gold standard methods for detection of ctDNA targets include droplet digital PCR and next-generation sequencing. However, those technologies require extraction of DNA from large volume of biofluid samples. EFIRM can be successfully used for continuous monitoring during treatment. The results are very promising [3].

Circulating tumor DNA (ctDNA) is considered to be stably found in biofluids encapsulated in extracellular vesicles (EVs) and released by cells into the circulation. If the links between distal

cancers and the oral cavity will appear to be scientifically proven, it will open a new avenue of clinical utility to effectively, and non-invasively diagnose cancers through saliva. The ctDNA mutant fragments were observed in plasma [69, 70] and saliva samples [71] of head and neck cancer patients.

3.2. Point-of-care technologies

The current knowledge of salivary biomarkers and their role in point-of-care applications highlights the need for development of more advanced technologies. As a consequence, point-of-care diagnostics is definitely approaching reality for salivary research and closely related with its translation into clinical practice [3] as it delivers information of the current status of the disease in a very fast, convenient and non-invasive way. PoCs can be successfully used for early detection and real-time monitoring of the disease [3].

The current PoC technologies are ubiquitous. They comprise microfluidics, micro/nanoelectromechanical systems (MEMS/NEMS), paper-based technology, RNA-sequencing, liquid biopsy, biosensors, fluorescent biosensors, photometric and electrochemical methods, electronic nose and electric field-based methods such as electric field-induced release and measurement (EFIRM) method [3, 68, 72]. Contemporary available PoCs can be delivered in form of small and portable smartphones or "lab-on-chips" [3].

One of such PoC development is the Oral Fluid NanoSensor Test (OFNASET), that is used for multiplex detection of salivary proteomic (thioredoxin and IL-8) and genomic biomarkers (messenger RNA biomarkers, i.e. SAT, ODZ, IL-8, and IL-1b) for oral cancer with 90% sensitivity and 90% specificity for both interleukin 8 (IL-8) and IL-8 protein messenger RNA (mRNA) [67]. In turn, OraRisk human papilloma virus (HPV) test with Reflex (Quest Diagnostics, Los Angeles, CA, USA) can be indicative of HPV infection, high risk factor for development of oral cancer [68]. In addition, electrical controlled magnetic EC Sensor is designed to detect microRNA-200a [73], electrochemical sensor using endonuclease target recycling amplification to capture oral cancer overexpressed 1 (ORAOV1) [74], while wireless mouthguard enzymatic biosensor to detect uric acid [75] or lactic acid [76], potential biomarkers for oral cancer.

4. Conclusions

Salivary diagnostics is a promising field for the implementation of PoC technology. The desire for PoC, the potential of saliva, development of validated panel of salivary biomarkers for specific diseases and development of novel advanced techniques enables the application of saliva for the early detection and diagnosis of several oral and systemic diseases in a non-invasive, easy and fast personalized way. The recent technology advances, including liquid biopsy, EFIRM, biosensors, smartphones, microfluidics, paper-based technology, have the potential to make clinical utilities of saliva a reality in the near future. Saliva is predicted to be a substitute for blood, collected non-invasively for the diagnosis of oral and systemic diseases as well as chairside screening.

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Conflict of interest

The author reports no conflict of interest in relation with the present study.

Notes/thanks/other declarations

Nothing to declare.

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